Antibody Solubility Behavior in Monovalent Salt Solutions Reveals Specific Anion Effects at Low Ionic Strength

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ABSTRACT: Protein solubility was measured using the crystalline precipitate of a recombinant therapeutic antibody, in monovalent salt solutions containing KF, KCl, and KSCN (up to ~0.7 M) at different pH conditions. For all three anions, the antibody solubility demonstrated complex behavior, both monotonic and nonmonotonic, with dependence on pH and salt concentration. At pH 7.1, close to the isoelectric point (p*I*) of 7.2, a typical salting-in behavior was observed with the salting-in constants of 12.7, 8.0, and 2.8 M for KSCN, KCl, and KF, respectively, suggesting that the anions follow the order of $SCN^- > Cl^- > F^-$ for increasing antibody solubility. Nonmonotonic behavior, as described by an initial solubility decrease followed by a solubility increase with ionic strength, was observed at pH 5.3, far below its p*I*. The effectiveness of the anion for reducing the solubility followed the order of $SCN^- > Cl^- > F^-$. After the solubility reached the minimum, the anion's effectiveness for raising the antibody solubility was in agreement with that at pH 7.1. The mechanisms for the above phenomena are discussed based upon specific binding of the anions to the antibody surface. The mechanistic view of anion binding and its charge neutralization effect at pH 5.3 was supported by the results from the effective charge and zeta-potential measurements. © 2011 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:965–977, 2012

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INTRODUCTION

Protein solubility is an important thermodynamic property of a protein solution. The solubility is defined as the protein concentration in a solution at equilibrium with its solid phase.[1] It can provide fundamental clues pertaining to the phase behavior of protein solutions described within a phase diagram such as crystallization, amorphous precipitation, and liquid–liquid phase separation (LLPS). Solubility is one of the key thermodynamic boundaries for understanding the effect of supersaturation and therefore designing rational approaches for protein crystallization. In the biopharmaceutical industry, it is important to understand protein solubility in order to design appropriate formulations to ensure long-term stability.

Salts play important roles in determining protein solubility, depending on the type, concentration, and the solution pH. It has been experimentally confirmed that a protein typically has low solubility at the pH condition near its isoelectric point (p*I*).[2,3] Empirically at pH near p*I*, salting-in (solubility increase) of proteins typically occurs as salts are initially added, and then salting-out (solubility decrease) happens as salt concentrations further increase.[1] It has been noted that the salting-out effect by anions follows the direct

Additional Supporting Information may be found in the online version of this article. Supporting Information

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Hofmeister series, that is, $SO_4^{2-} > H_2PO_4^- > F^- > Cl^- > Br^- > I^- > SCN^-$, when pH is above the p*I* of a protein.[4–8] But the monovalent anion's effect for reducing protein solubility abides by an inverse Hofmeister series, that is, $SCN^- > Cl^- > F^-$ when pH is below the p*I*.[7]

Monoclonal antibody solubility in salt solutions is still poorly understood and there is a lack of systematic experimental studies to evaluate the perturbation of pH, ionic strength, and salt type on monoclonal antibody solubility. In this article, we measured the solubility of a monoclonal antibody at room temperature, using its crystalline precipitate, in the monovalent salt solutions of KF, KCl, and KSCN at different ionic strengths under three pH conditions. The equilibrium antibody concentration in the aqueous phase during the solubilization experiments was reported as the antibody solubility in our study. It should be pointed out that the measured solubility might not be the thermodynamic solubility for this antibody because its crystal polymorphism has not been characterized.

It is our objective to understand how the incremental change in monovalent anion concentrations affects the antibody solubility in a common pH range for typical protein liquid formulations and salt concentrations up to the physiological level, where the specific ion–protein interactions may be more important than the Hofmeister series effect. The Hofmeister series effects are typically used for explaining protein solution behavior at moderate-to-high (>0.3 M) salt concentration when the repulsive electrostatic intermolecular interactions are completely screened.[4,9,10] We chose the above three anions because they follow the order of $F^- > Cl^- > SCN^-$ for precipitating proteins according to the (direct) Hofmeister series.[4–8] Although the exact interaction mechanisms of the electrolyte ions with proteins remain unclear,[11–14] the above three monovalent anions should perturb solubility of the antibody as a function of pH and ionic strength because they rank from the strongly hydrated F^- to the weakly hydrated SCN^-.[4–8] Our experimental results demonstrate that the anions could switch their ranking of effectiveness for affecting the antibody solubility before and after the completion of charge neutralization at one given pH below its p*I*. The behavior indicates the presence of specific interactions between the anion and the antibody. The above trends agree with the protein–protein interaction behavior for this antibody in the same set of salt solution conditions as revealed by the LLPS.[15] It is anticipated that this work may fundamentally contribute to an understanding of how salts affect the solubility of monoclonal antibodies and, in addition, augment the current knowledge pertaining to protein solubility in different salt solutions.

MATERIALS AND METHODS

Preparation of Antibody Crystalline Precipitate Solution

The antibody solution at approximately 70 mg/mL in 10 mM sodium acetate (pH 5.2) and 9% sucrose (A52Su) was produced at Amgen, Inc. (Thousand Oaks, California) and dialyzed into a 0.2 mM KSCN solution using a 10 kDa molecular weight cut off (MWCO) slide-a-lyzer (Pierce, Rockford, Illinois). The antibody is an immunoglobulin G2 molecule with an experimentally measured p*I* of 7.2. The dialysis was performed at 2°C–8°C over 48 h with five exchanges and a volume ratio of 1:100 for every exchange. After dialysis, the protein was concentrated to approximately 80.3 mg/mL using an Amicon 15 centrifugal device (Millipore, Billerica, Massachusetts) (10 kDa MWCO). The concentrated protein solution was filtered in a sterile biosafety hood with a 0.2 μm cellulose acetate membrane filter device. Sterile potassium phosphate (dibasic, 1.0 M) was then added to the protein solution to reach pH 7.1 ± 0.1 and approximately 10 mM potassium phosphate (ionic strength). The antibody started to crystallize within a few minutes after the addition of potassium phosphate. The solution was kept at room temperature for 3 days before transferring to 2°C–8°C for storage.

Microscope Procedures

An aliquot of the sample was taken and laid on top of a 22 mm siliconized glass cover slide. The sample was inspected with a Zeiss Stemi SV11 stereomicroscope with polarization (Carl Zeiss Microscopy LLC, Thornwood, New York), which was interfaced with a Zeiss AxioCam MRc digital camera operated with AxioVision 4.0 software to take the color photographs. The magnification was 105.6× by using Zeiss W-P1 eyepieces 16×/16 with a zoom of 6.6×.

Supersaturation Tests for the Solubilized Antibody Solution at pH 5.3

The design of the experiments is shown in Figure 1. The crystalline precipitate solution from above was centrifuged at 3000 rpm (~ 825 g) for approximately 15 min and the supernatant was removed. After having washed twice with deionized water, the pellet was dissolved in the solubilization media of the pH 5.3 starting solution by a combination of gentle constant vortexing and shaking at day 0, and then remained gently shaken on a horizontally rotating table. To ensure that the protein concentration was close to the solubility, we added only enough solubilization media to dissolve the majority of the antibody crystals with a remnant of crystals at the bottom of the tube after centrifugation. Four different experiments were conducted to verify supersaturation in the supernatant and discern the formation of a new solid phase. In

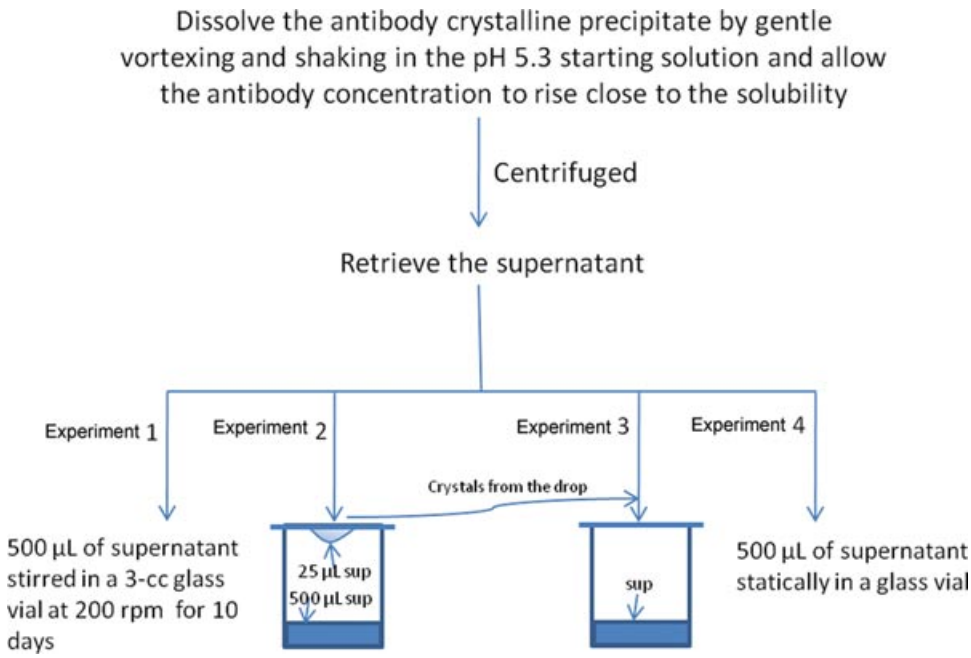

Figure 1. Illustration for conducting the supersaturation tests using the supernatant obtained from the dissolution of the crystalline precipitate in the pH 5.3 starting solution. "sup" is the abbreviation for supernatant.

Experiment 1, the supernatant was stirred with a magnetic bar in a 3-cc glass vial for 10 days to accelerate the generation of the crystals. The antibody concentration was monitored, and an aliquot of the solution was inspected under the microscope to discern the presence of amorphous or crystalline phases. In Experiment 2, the supernatant was used for crystallization in a traditional hanging-drop format with 500 µL of the supernatant added into the bottom of the well and a drop of 25 µL of the supernatant on the underside of the cover slide at the top of the well. In Experiment 3, a small amount of crystals from the hanging drop was seeded into the supernatant to confirm the supersaturation. In Experiment 4, 500 µL of the supernatant was incubated in a 3-cc glass vial statically stored to test for long-term crystallization and confirm supersaturation. All the above experiments were conducted at room temperature.

Antibody Solubility Measurements

Solubilization Media Preparation

Three pH buffer solutions at pH 5.3, 6.1, and 7.1 with a calculated 22 ± 1 mM ionic strength (they are referred as the pH 5.3, 6.1, and 7.1 starting solutions, respectively) were prepared similarly as previously described.[15] The pH of the solution was then verified to be within ±0.1 of the above designated value by a Thermo Orion pH meter with a Thermo Micro Combination pH electrode (Fisher Scientific, Pittsburgh, Pennsylvania). The buffered solutions were sterile filtered. Then, the solubilization media comprising KF, KCl, or KSCN at the three pH conditions were prepared by adding appropriate amounts of each salt from their respective sterile stock solutions to the pH starting solutions. Thus, the introduction of salt would add to the buffer solution ionic strength. The volume of the salt stock solution added was no more than 10% of the pH buffer solution's volume. All the solubilization media were stored at 2°C–8°C before use. The salt concentrations reported are the total ionic strength in the solubilization media, including the buffer salts, unless otherwise noted. The ionic strength was then calculated based on the following equation:

$$\mathrm{IS} = \frac{1}{2}\sum_{1}^{n} C_i Z_i \qquad (1)$$

where C_i is the molar concentration of ion i, Z_i is the charge number of that ion, and the sum is taken including all ions in the solution.

Solubility Plate Design and Preparation for Concentration Measurement

The antibody crystalline precipitate solution (550 µL) was manually added into the wells of a Symyx 96 well plate (Symyx Technologies, Santa Clara, California), then sealed and centrifuged. The details of the plate design have been described previously.[16] Using a robotic liquid handling system (Symyx Technologies), the supernatant was removed and 700 µL of sterile water was added to each well to wash the pellet. The

plate was inverted several times and centrifuged prior to removal of water using the liquid handler. The water washing step was performed twice. Finally, 300 μL of solubilization media were added to each well. The robotic system was rinsed with sterile water before a different solution was used. After the plate was centrifuged, the samples were ready for the initial ($t0$) protein concentration measurements. The plate remained gently shaken on the horizontally rotating table for solubilization when not used for concentration measurement. During the entire study, the samples were stored at room temperature.

Concentration Measurement by Size-Exclusion Chromatography

A 2-μL aliquot of the supernatant was sampled for the antibody concentration measurement by a 10-min size-exclusion chromatography (SEC) method using the Agilent 1200 high-performance liquid chromatography (HPLC) system equipped with a Symyx 96-well plate autosampler module (Agilent Technologies, Santa Clara, California). The SEC method was operated at a flow rate of 1.2 mL/min under isocratic conditions with 100 mM sodium phosphate and 250 mM sodium chloride at pH 6.8 using a Zenix SEC-300 column (7.8 × 300 mm, 3 μm) from Sepax Technologies (Newark, Delaware). Both the solubility samples and antibody standard solutions were subjected to SEC analysis. The areas of the main peaks from the standards were used to derive a standard curve by linear regression analysis. Using the standard curve, the antibody concentration of each sample was determined. The protein monomer peak eluted at approximately 6.7 min (monitored by ultraviolet detection at 280 nm). A control at 14 mg/mL was used to demonstrate the HPLC system suitability during the concentration measurement. It was analyzed for each run at the day of testing. The wells were inspected under the microscope at the end of the experiment, and the solubility was only reported for those conditions still containing the precipitates.

Data Analysis of Antibody Solubility at pH 7.1

The antibody solubility in the KF, KCl, and KSCN solutions at pH 7.1 was fitted by the Setchenow equation shown below[4]:

$$\log[C_i/C_i(0)] = k_s C_s \tag{2}$$

where C_i is the solubility of a compound (i.e., protein) at a specific salt concentration, $C_i(0)$ is the solubility of the compound without the salt, C_s is the molar concentration of the salt, and k_s, if positive, is the salting-in constant (if negative then refers to salting-out). The solubility in the pH 7.1 starting solution was used as $C_i(0)$.

Electrophoretic Mobility Measurements

We employed a combination of electrophoretic mobility and sedimentation velocity experiments to determine the effective charge (Q^*) of the antibody in units of elementary charge, e. The method, as executed in our laboratory, has been detailed previously[17] and relies on the three relationships (Eqs.3–5). The Q^* of the antibody at a given temperature (T) is related to its electrophoretic mobility (μ), translational diffusion coefficient (D_t), and Boltzmann's constant (k_B). Electrophoretic mobility (μ) was determined using capillary electrophoresis with a Beckman Coulter PA 800 instrument (Beckman Coulter Inc., Brea, California) and a Beckmann Coulter eCAP™ Amine Capillary [60 cm total length (C_t); 50 cm to detector (C_d)]. The mobility was derived from the elution time of the antibody (t_{mAb}) and a neutral electroosmotic flow (EOF) marker (t_{EOF}, 0.005% benzyl alcohol). The antibody was buffer exchanged using a GE Healthcare (Piscataway, New Jersey) NAP™-5 into 20 mM acetate, pH 5.3, with a final antibody concentration of approximately 35 mg/mL. This material was subsequently diluted in appropriate salt/buffer solutions to 1 mg/mL. Hydrodynamic injection (0.5 psi for 3 s) was used to load both the EOF marker and protein (in that order) onto the column. The applied potential (P) ranged from 11 to 21 kV; four voltages were used for each μ determination (e.g., 11, 13, 15, and 17 kV). The final component, the diffusion coefficient (D_t), was derived using the sedimentation coefficient (s), universal gas constant (R), molecular weight (M_w), partial specific volume ($\bar{v}$), and solvent density (ρ). The value of s was determined by sedimentation velocity analytical ultracentrifugation (SV-AUC) of 1 mg/mL antibody using the absorbance detection system of a Beckmann Coulter XL-I (Beckman Coulter Inc.). Sedfit v12[18] was used to perform analyses and to calculate the average s value of the antibody in three solutions: 20 mM acetate, pH 5.3, with (1) no added salt, (2) 75 mM KCl, and (3) 75 mM KSCN. The resulting values were 6.6, 6.6, and 6.7 s, respectively. Consistent with our previous analysis of lysozyme, the average value (6.6 s) was used throughout our calculations. The values of ρ were obtained using the program SEDNTERP v. 1.09.43 or through measuring protein-free salt solutions in a DMA 5000 densitometer (Anton Paar GmbH, Graz, Austria), calibrated to air and water.

$$Q^* = \frac{\mu \times k_B \times T}{D_t e} \tag{3}$$

$$\mu = \left(\frac{C_d}{t_{EOF}} - \frac{C_d}{t_{mAb}}\right)\left(\frac{C_t}{P}\right) \tag{4}$$

$$D_t = \frac{sRT}{M_w(1 - \bar{v}\rho)} \tag{5}$$

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Zeta-Potential Measurements

Zeta potential values of the antibody samples were measured with a Malvern Zetasizer Nano S instrument (Malvern Instruments Ltd., Worcestershire, UK) at 25°C using DTS 1070 zeta dip cell with 12 mm square glass cuvette. The zeta potential was calculated by the DTS (nano, ver.5.03) program. The samples were prepared at 3.0 mg/mL in different salt solutions at pH 5.0 buffered with 10 mM sodium acetate, and then measured directly. Triplicate measurements were made for each sample and the averaged zeta potential value was reported.

RESULTS

Kinetics Data during Solubilization at pH 5.3

Shown in Figure 2 are the kinetic data during the solubility measurement in various KSCN ionic strengths at pH 5.3 up to 25 days. It generally took almost 14 days to reach the final equilibrium concentration for the low-solubility condition, whereas it took only 4 days for some high-solubility conditions. It was obvious that low solubility translated into long equilibrium times. The slow kinetics for reaching the equilibrium might be due to the 10-h static storage of the plate in the HPLC system during the concentration measurement.

It is interesting to note that for some of the solution conditions at pH 5.3, for example, the starting solution without the addition of salt (0 mM in Fig. 2), a gradual decrease in the antibody concentration over time was observed after a maximum antibody concentration was reached. We did not see this type of behavior in the KSCN series at pH 6.1 and 7.1 (Figs. IIIa and IIIb in Supporting Information). In both KF and KCl series, the slow drop in protein concentration occurred as well at pH 5.3 (Figs. IVa and Va, respectively, in Supporting Information), but not at pH 6.1 and 7.1 (Figs. IVb and IVc, and Figs. Vb and Vc in Supporting Information). In a separate study, the antibody at pH 5.3 was tested by cation exchange chromatography and the results did not show the appearance of significant antibody degradation.

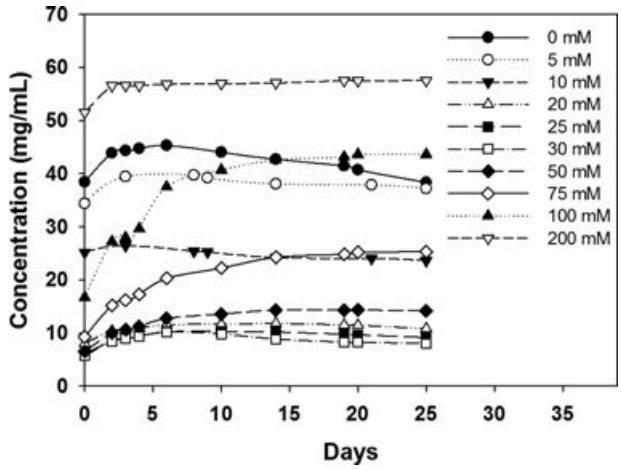

Figure 2. Solubilization data for the KSCN series at pH 5.3 buffered with 22 mM sodium acetate. Only the amount of KSCN added is depicted in the legend, but it is understood not to be the total ionic strength of the solutions because it is does not include the initial 22 mM ionic strength provided by the buffered solutions. Similar solubilization kinetics was observed for the KCl and KF series at pH 5.3 (data not shown).

We performed a series of experiments as described in Figure 1 to investigate the formation of a different or new solid phase to explain the concentration decline at pH 5.3. The crystalline precipitate obtained at pH 7.1 was dissolved in the pH 5.3 starting solution as described in the subsection *Supersaturation Tests for the Solubilized Antibody Solution at pH 5.3* under the section *Materials and Methods*. As shown in Figure 3a, the antibody concentration decreased over time from approximately 46 to approximately 29 mg/mL, consistent with the observed concentration decrease during the solubility measurement. The later fast drop in protein concentration in Figure 3a might be caused by the stirring. At the end of the 10-day stirring experiment, we observed very small crystal-like particles, that is, less than 50 μm (Fig. 3a, inlay). In Experiment 2 as described in Figure 1, the hanging-drop method would encourage crystallization due to solution evaporation. Indeed, we observed crystal formation in the drop within a few days as shown in Figure 3b, but not in the well. The shape of this crystal was needle like and distinctively different from that obtained at pH 7.1 (Fig. I in Supporting Information). In Experiment 3, massive crystallization was observed in the well within a day (data not shown) after we seeded the solution in the well with the crystal from the drop, suggesting that the supernatant was supersaturated with respect to the crystal formed in the drop. In Experiment 4, a small amount of crystalline form (Fig. 3c) was found at the bottom of the vial after 50 days of incubation. The results suggest that this crystallization has a slow kinetics without the assistance of stirring, solution evaporation, or seeding.

For those samples showing a decrease in concentration after reaching a plateau, the average value calculated from the maximum concentration together with those immediately before and after was reported as the approximate solubility of the crystal precipitate. The initial fast rise and the later gradual decline of the antibody concentration in those instances suggested that the kinetics of the antibody solubilization process was faster than the formation of a

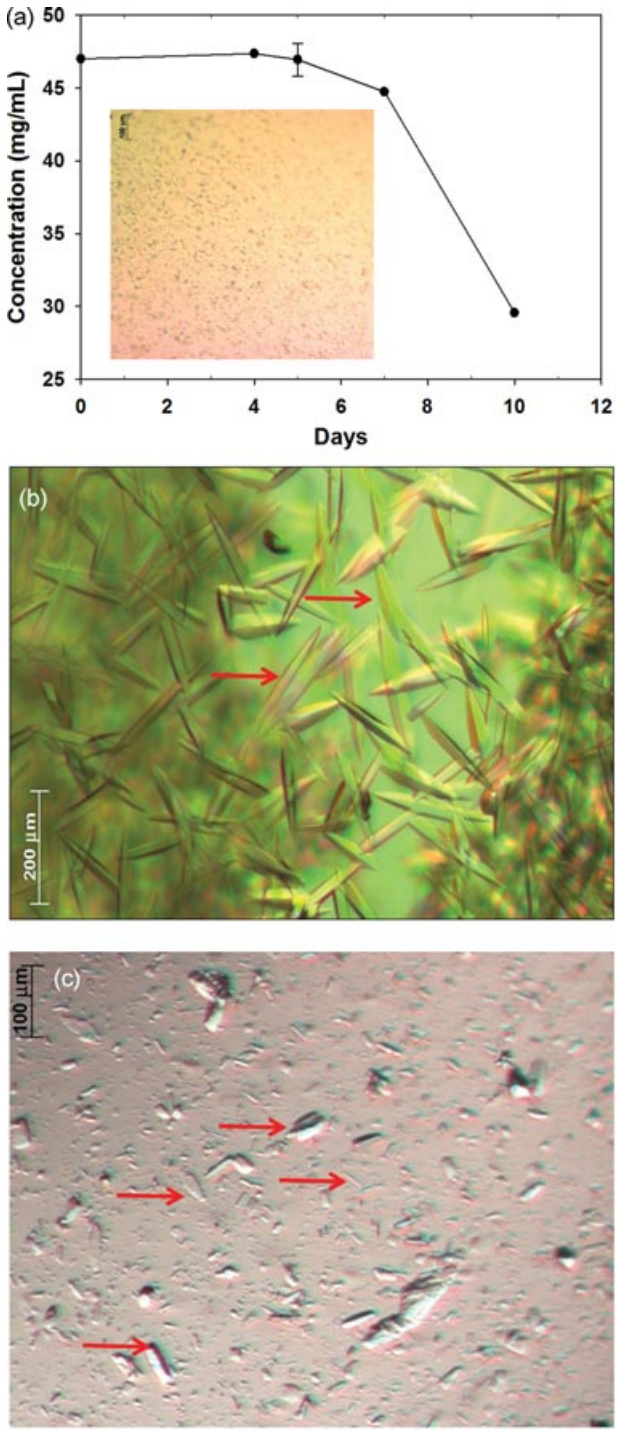

Figure 3. (a) Monitoring of the antibody concentration in the supernatant during stirring. Insert is the partial view of the microscope image of the solution at the end of Experiment 1. Four independent concentration measurements were performed at day 5 to establish the precision of the measurement. (b) Partial view of the microscope image for the drop after crystallization of the resolubilized antibody in the pH 5.3 starting solution, using the crystalline precipitate from pH 7.1 in a hanging-drop format as described in Experiment 2. (c) Partial view of the microscope image for the solution after 50 days in Experiment 4. Red arrows point to the representative crystals in the samples.

new solid phase, which was confirmed by the results of Experiment 1, as shown in Figure 3a: the protein concentration began to drop at day 5. Therefore, the above approach could be used to approximate the equilibrium concentration amidst a gradual decrease during the time course of the experiment. For those samples that did not show a decrease in antibody concentration within 25 days, the average of the final three measured concentrations was reported as the solubility.

Antibody Solubility at pH 7.1

Figure 4a shows the solubility response as a consequence of increasing ionic strength at pH 7.1, close to the p*I* of the antibody. The solubility increased as the ionic strength rose, which is typical of salting-in behavior for the three salts. At pH 7.1, the antibody

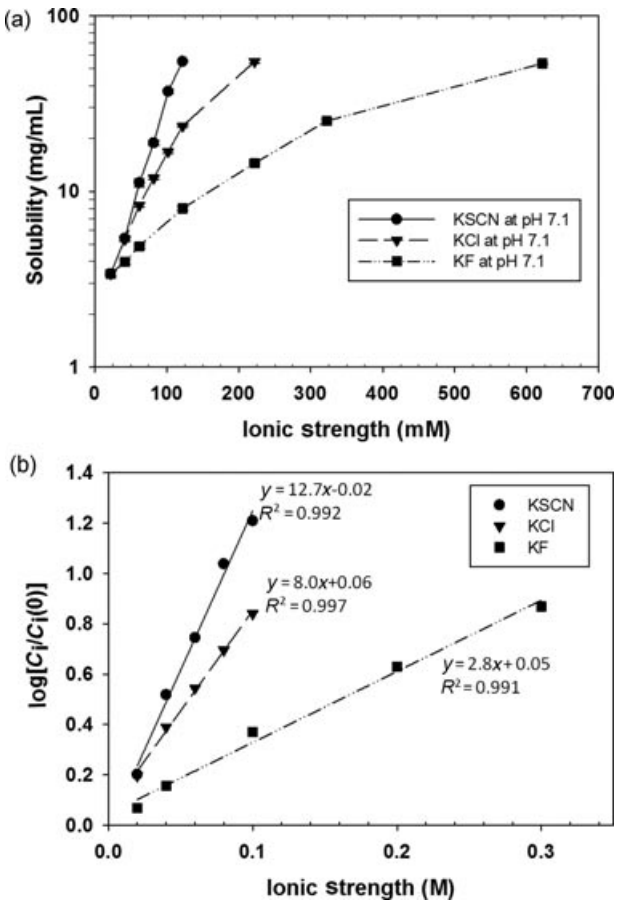

Figure 4. (a) Solubility at pH 7.1 buffered with 22 mM potassium phosphate versus the total ionic strength with the respective addition of KSCN, KCl, and KF solutions. The lines were drawn to guide visual evaluation. (b) Setchenow plots for the KSCN, KCl, and KF series at pH 7.1. For the KCl and KF series, higher ionic strength solubility data were not included because of the onset of salting-out effect. The solubility in the pH 7.1 starting solution was used as the solubility $C_i(0)$.

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had the highest solubility in the KSCN solution relatively to KCl or KF at the same ionic strength. The Setchenow plots for the three salts are shown in Figure 4b for the addition of KSCN, KCl, and KF up to 0.1, 0.1, and 0.3 M, respectively. The calculated salting-in constants from the plotted results were found to be 12.7, 8.0, and 2.8 M^{-1} for KSCN, KCl, and KF, respectively. Therefore, the salting-in strength for the three anions follows the order of $SCN^- > Cl^- > F^-$.

If the solubility data at 0.2 M KCl and 0.6 M KF were included, a loss in linear fit was observed. The onset of loss of linear fit for KCl and KF at 0.125 M KCl and 0.3 M KF suggests the presence of a salting-out effect. However, no apparent salting-out effect was observed for KSCN.

Antibody Solubility in KF, KCl, and KSCN at pH 5.3

In Figure 5a at pH 5.3, there is a nonmonotonic relationship between antibody solubility and ionic strength in the three salt solutions. As the ionic strength increased, the solubility decreased, reached a minimum, and then rose again.

The solubility was most sensitive to the ionic strength increment of SCN^-, followed by Cl^- and F^-. In Figure 5a, the addition of approximately 20 mM KSCN decreased the solubility from approximately 45 to approximately 9.0 mg/mL, whereas the addition of approximately 200 mM KF lowered the solubility from approximately 45 to approximately 23 mg/mL. Therefore, KSCN most effectively decreased the antibody solubility. This type of behavior was reported for lysozyme in monovalent salts as well.[19]

In Figure 5a, the antibody solubility minimum in the KSCN series was much lower, that is, approximately 9 mg/mL, than both KCl and KF, that is, between 20 and 30 mg/mL. Additionally, the minimum was reached at different ionic strength: approximately 50 mM in the KSCN series, followed by approximately 70 mM in the KCl series, and approximately 220 mM in the KF series.

A further increase in ionic strength beyond the antibody solubility minimum led to an increase in antibody solubility (salting-in) for all salts in Figure 5a. The rise in antibody solubility was steepest for the KSCN series, followed by KCl and KF. This trend is similar to observations at pH 7.1, which indicated the strong salting-in effect by SCN^- in contrast to the weak salting-in effect by F^-.

At pH 6.1 as shown in Figure 5b, the nonmonotonic behavior could only be observed for the KSCN series. For the KCl and KF series, the antibody solubility increased with ionic strength. But the change in solubility at pH 6.1 appeared to be less sensitive to ionic strength up to approximately 100 mM than at pH 7.1. As indicated in Figure VIa from Supporting Information, the addition of 100 mM KCl to the 22 mM potassium phosphate solution increased the solubility by approximately sixfold at pH 7.1, whereas the same amount increased the solubility by approximately fourfold at pH 6.1. Similar behavior was observed for KF, as shown in Figure VIb from Supporting Information.

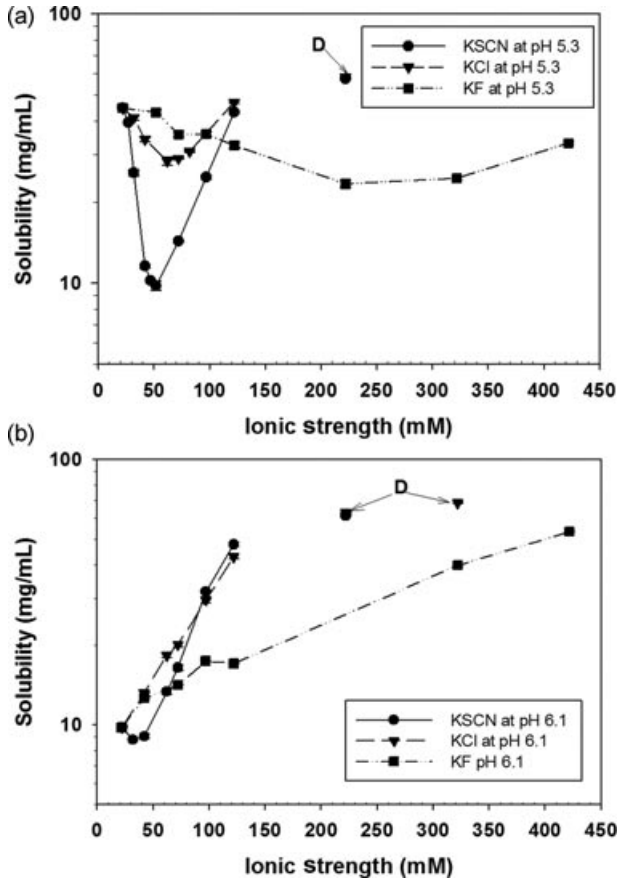

Figure 5. Solubility versus the total ionic strength in the solution with the respective addition of KSCN, KCl, and KF, including the buffer salt: (a) at pH 5.3 buffered by 22 mM sodium acetate. At 222 mM in the KCl and KSCN series, all the pellets were dissolved; (b) at pH 6.1 buffered by 22 mM potassium phosphate. In the KSCN series, at 222 mM, all the pellets were dissolved. In the KCl series, at 222 and 322 mM, all the pellets were dissolved. "D" means that the pellet was all dissolved and therefore the concentration cannot be used as the final solubility. The lines were drawn to guide visual evaluation.

Electrophoretic Mobility and Zeta-Potential Measurement

The Q^* values derived for the antibody were consistent with those previously reported for similar monoclonal antibodies.[20] The Q^* decreased as a function of salt concentration, from a value of approximately 3.9e in the base formulation to 1.8e in the presence of 75 mM KCl (Fig. 6a). An anion-specific effect was

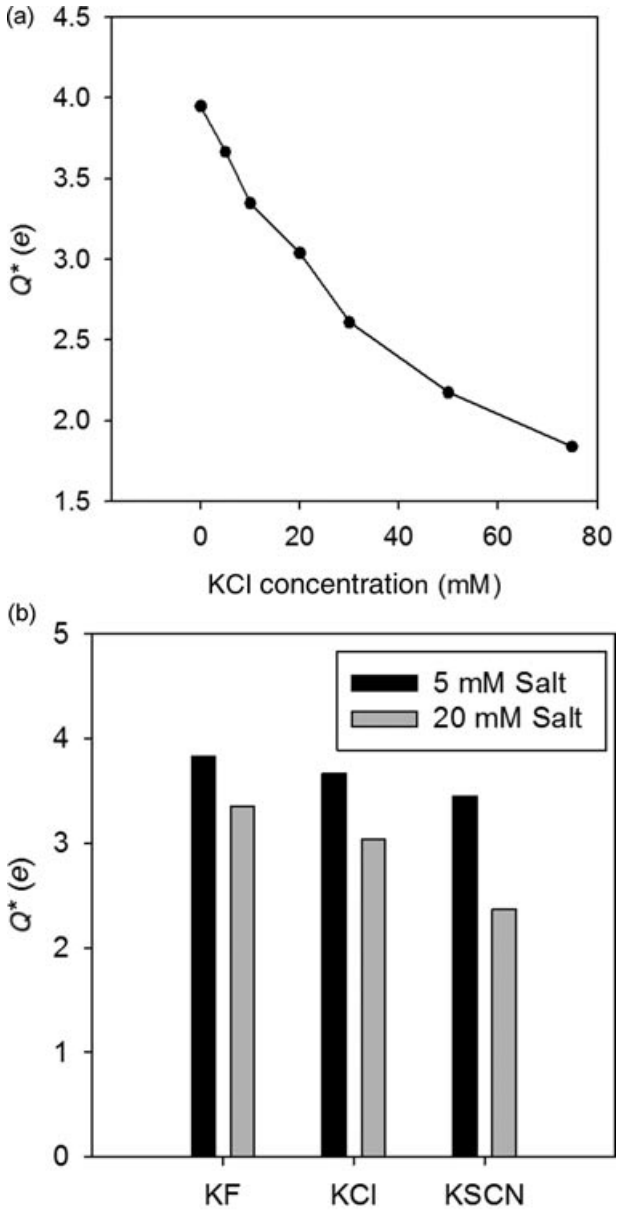

Figure 6. Q^* as function of salt. (a) The Q^* of the antibody was sensitive to KCl concentration, falling as a function of increasing salt concentration. The lines were drawn to guide visual evaluation. (b) The same effect was observed with KF and KSCN; however, the antibody was sensitive to the identity of the anion, showing less reduction with KF and more with KSCN.

observed, as previously described for both antibodies[20] and lysozyme.[17] A kosmotropic anion, F^-, caused a smaller reduction in Q^* as a function of concentration, whereas a more chaotropic anion, SCN^-, displayed a larger reduction (Fig. 6b). As shown in the Figure 7, the zeta potential generally decreased when the salts were added. In addition, KSCN was most effective for decreasing the zeta potential, whereas KF was least effective.

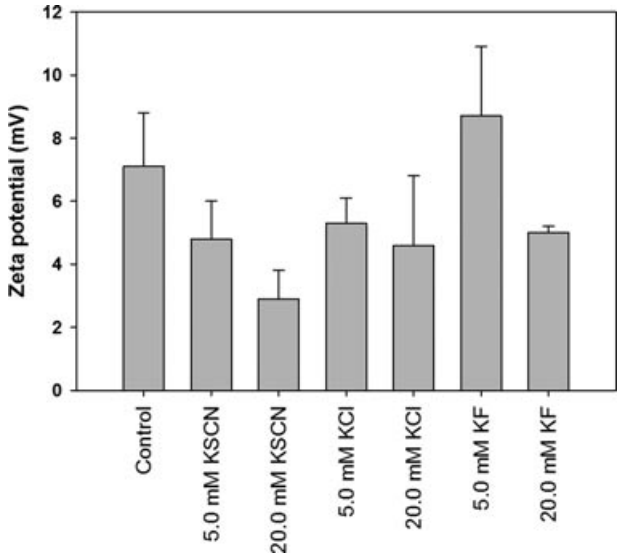

Figure 7. Measurement of zeta potential in 5.0 and 20 mM of KF, KCl, and KSCN solutions, respectively, at pH 5.0.

DISCUSSION

Antibody Crystal Polymorphism

The apparent decline (Fig. 3a) in protein concentration at pH 5.3 during the measurement suggested that there may be a formation of a new solid phase specific to the pH 5.3 condition as a result of supersaturation after dissolution of the antibody crystalline precipitate from pH 7.1. In Experiment 1 as described in Figure 1, the stirring of the supernatant accelerated the formation of small crystals. Crystallization in the drop in Experiment 2 indicates that the antibody solution was supersaturated with respect to the new crystals formed. In addition, the massive crystallization from the seeding experiment in Experiment 3 provided direct evidence of supersaturation. The slow crystallization observed in Experiment 4 was another evidence of supersaturation. Overall, these four experiments suggested that after the crystal from pH 7.1 (neutral pH crystal) was dissolved in the pH 5.3 starting solution, the antibody solution at equilibrium was supersaturated with respect to the crystal obtained from the above hanging-drop experiment (acidic pH crystal). It might be inferred that the solubility of the neutral pH crystal is higher than that of the acidic pH crystal in the pH 5.3 starting solution. Thus, it might be possible for the dissolved antibody to form a new solid phase, resulting in the concentration decrease observed during the solubility experiment. The solubility difference between the two crystal forms (acidic and neutral pH forms) might be the experimental evidence for the presence of crystal polymorphs for this antibody. Solubility differences

between crystal polymorphs have been reported previously for urate oxidase[21] and aprotinin.[22]

Protein crystal polymorphism is still poorly understood. The observation of the possible crystal polymorphs for this antibody suggests that protein crystal polymorphs might be related to the counter-ion binding to a net-charged protein. According to the theory that only a net neutral species can undergo a phase change,[23,24] it is anticipated that the amount of salt ions incorporated into the neutral pH crystal should be low because the antibody was charged neutral at pH 7.1, close to its p*I* of 7.2. After the charge-neutral antibody crystal was dissolved in the buffer or in the salt solutions at a different pH condition, that is, 5.3, the antibody molecule became net positive-charged. The counterions either from the buffer and/or the salts began to interact with the antibody and form a counterion–antibody complex. At the conditions of supersaturation, the complex crystallized out of solution. The idea appears to be consistent with our experimental observation.

Thermodynamic Considerations for Protein Solubility in Salt Solutions

At equilibrium during protein solubility measurement, the chemical potential of a protein in the solution phase is equal with that in the solid phase as described by Timasheff[25]:

$$\mu_2^{\mathrm{l}} = \mu_2^{\mathrm{s}} \tag{6}$$

where μ is the chemical potential, and 2, l, and s refer to the protein, liquid phase, and solid phase, respectively. The chemical potential of the protein in the solution phase can be further described by the following equation:

$$\mu_2^{\mathrm{l}} = \mu_2^0 + RT\ln(\gamma_2 m_2) \tag{7}$$

where μ_2^0 is the standard chemical potential of the protein including all the protein–solvent interaction free energy, γ_2 and m_2 are the protein's activity coefficient and molality in the equilibrium solution, respectively. Protein–protein interactions are included in γ_2. Apparently, change in protein solubility as reflected by m_2 is caused by the free energy differences from both solvent–protein interactions and protein–protein interactions. The assumption for the above scenario is that the chemical potential of the protein in the solid phase is independent of the solution phase change.[25]

Anion-Specific Salting-In of Antibody at pH 7.1, Close to its p*I*

The above thermodynamic consideration can be further expanded to draw the attentions to solvent–protein interaction free energy, under the condition wherein γ_2 is assumed to be relatively constant.[25] This condition might be valid for a net-charge neutral or weakly charged protein, that is, near its p*I*. It should be mentioned that there are many theoretical models to rationalize protein solubility behavior in salt solutions.[1,26] In essence, the electrostatic interactions and hydrophobic interactions are the two major elements for consideration.[1,26] The effects from the electrostatic interactions on the free energy of a protein in salt solutions may be described by Debye–Hückel theory in combination with Kirkwood's expression of protein dipole moment as follows[1,26]:

$$\Delta G_{\mathrm{e.s.}} = A - \frac{B(I^{1/2})}{1 + C(I^{1/2})} - DdI \tag{8}$$

where A, B, C, and D are constants; I is the ionic strength of the solution; and d is the dipole moment for the protein. This theory predicts the salting-in effect: as the salt concentration increases, protein solubility rises. The main limitation of this theory is that it does not consider ion specificity. The free energy change for a protein involving the hydrophobic interactions may be illustrated by the cavity theory as follows[1]:

$$\Delta G_{\mathrm{cav}} = \left[N \times \mathrm{Area} + 4.8N^{1/3}(k^e - 1)V^{2/3}\right]\left(\frac{\partial\sigma}{\partial m_3}\right)m_3 \tag{9}$$

where N is Avogadro's number, Area is the surface area of a protein molecule, k^e corrects the macroscopic surface tension if the solvent to molecular dimensions, V is the protein's molar volume, $\left(\frac{\partial\sigma}{\partial m_3}\right)$ is the molal surface tension increment of the salt, and m_3 is the molality of the salt. This cavity theory describes how much free energy is needed to form a cavity in the solution to accommodate a hydrophobic protein molecule. Therefore, the surface tension of the solution is an important parameter and its modulation by salts impacts protein solubility. It predicts that the addition of kosmotropic salts, which increase the solution surface tension, will result in the salting-out effect. Therefore, these salting-in and salting-out effects in combination modulate protein solubility in salt solutions.[1,26] Specifically, near p*I*, the salting-in effect dominates initially. Then, further increase in (kosmotropic) salt concentration results in a transition to the drop in protein solubility as the salting-out effect begins to dominate.

Salt ions could interact with protein surface through at least four possible mechanisms. Near p*I*, there are positive- and negative-charged side chains exposed to the solvent that can provide interaction sites for the counterions. In Mechanism I, there could

be ion binding to protein surface caused by direct interactions between the counterions and protein-surface-charged residues.[27,28] Alternatively, in Mechanism II, the anion could favorably interact with the peptide groups through the nitrogen atom.[4,6,8,29,30] Additionally, in Mechanism III, the kosmotropic anion could polarize water molecules hydrogen-bonded with the amide bonds exposed on the surface of proteins.[8] Finally, in Mechanism IV, both chaotropic and kosmotropic anions could desolvate surface-exposed nonpolar groups, thereby reducing the entropic penalty that arises from hydrophobic hydration.[8]

It is generally accepted that for a net-charge neutral protein at a pH close to the p*I*, the first two mechanisms lead to the salting-in effect for proteins, whereas the latter two result in the salting-out effect.[1,31–36] In our discussion, "salting-in effect" refers to the net effect of the first two mechanisms, consistent with the effect of the electrostatic interactions as described in Eq. 8, whereas "salting-out effect" refers to the latter two, in agreement with the hydrophobic interactions as presented in Eq. 9. As will be presented later, a decrease in solubility can also happen because of charge neutralization. It is further pointed out that the salting-in effect rises exponentially as a result of the binding-saturation behavior, whereas the salting-out effect increases linearly with salt concentration.[8] Therefore, it is generally expected that as the salt concentration increases, the salting-in effect initially dominates and protein solubility rises sharply. Then, the salting-out effect (for kosmotropic salts) begin to compete and impede the solubility rise. Eventually, protein solubility drops as the salting-out effect takes over. In summary, the impacts on protein solubility from the above four possible mechanisms are consistent with those from thermodynamic considerations.

Our observation of the initial rise of the antibody solubility up to 0.1 M in the three salt solutions is in agreement with the above salting-in notion for a net-charge neutral protein, which is the case for the antibody at pH 7.1, close to its p*I*. This indicates that mechanistically the anions may interact with the antibody and bind to its surface. Then, the later loss of the linear fit in the KCl and KF series at approximately 125 and approximately 300 mM, respectively, suggests the hindrance of the increase in antibody solubility by the salting-out effect. Overall, the increase in the antibody solubility data in all the three salts suggests that the salting-in effect from the interactions as defined by Mechanisms I and II dominates the salting-out effect by Mechanisms III and IV up to 700 mM.

In addition to the general trending of initial solubility increase by the salts, the difference in the salting-in constant for the three salts clearly demonstrates an anion-specific effect on the antibody solubility. The effect from K^+ is not considered here because it was a common cation used in our study and its effect should then be normalized. Specifically, the weakly hydrated SCN^- (chaotropic) is more effective at raising the antibody solubility than the strongly hydrated F^- (kosmotropic). The anion-specific behavior cannot be explained by the electrostatic interactions as defined by the Debye–Hückel theory because it only accounts the valency of an ion for its interaction with proteins. The anion-specific behavior suggests that the binding between SCN^- and the antibody may be the strongest, followed by Cl^- and F^-. The above trends agree with the findings of our previous work in the protein–protein interaction measurements using the LLPS for this antibody at pH 7.1 in the same set of salt solutions.[15] This idea is consistent with the recent findings wherein a chaotropic monovalent anion binds more strongly to a net-charge neutral macromolecule, such as Bovine Serum Albumin (BSA) near its p*I*[37] and polar poly-(N-isopropylacrylamide),[38] than a kosmotropic monovalent anion. In fact, the ranking for the binding strength (which can be defined by binding constant) between the monovalent anions and the antibody is in agreement with that as defined by the "law of matching water affinities": namely, ion pairs are preferentially formed between opposite charged ions with matching absolute enthalpies of hydration.[6,7,24] For example, the weakly hydrated SCN^- should have the strongest interaction with the antibody through the weakly hydrated positive-charged side chains of lysine, arginine, histidine, and possibly peptide group, whereas the strongly hydrated F^- should be the weakest.[6,7,24] This notion is in agreement with our experimental findings.

Charge Neutralization by Specific Anion Binding at pH below p*I*

At pH 5.3, substantially lower than the p*I*, as ionic strength increased, the solubility decreased, reached a minimum, and then rose. The initial decrease in solubility by the three salts is in agreement with the lysozyme solubility data.[19,39] It was shown that a monotonic decrease in solubility versus ionic strength increase was observed for lysozyme over a wide pH range from 9 to 3 for a NaCl solution between 0.05 and 1.2 M.[39] Also, it was reported that the strong chaotropic anion, that is, SCN^-, was more effective at reducing the solubility of lysozyme at pH 4.5 than the weak chaotropic anion, for example, Cl^-.[19]

The results from the Q^* and zeta-potential measurement confirm that the antibody is positively charged at pH 5.3, below its p*I*. The reductions in Q^* and zeta potential as a function of salt concentration are the results of the antibody–solvent interaction, suggesting that mechanistically the anions bind to the positive-charged antibody surface and neutralize the net charges. The presence of solubility minimum

indicates that the anions complete their neutralization process and Q^* reaches a minimum. Our results are consistent with Tanford's[40] theory that the protein minimum solubility is reached when the protein binds an equal number of counterions to saturate its surface charge.

For a net-positive-charged protein, such as this antibody at pH 5.3, at low ionic strength below approximately 100–200 mM, it is generally recognized that there are repulsive electrostatic interactions between protein molecules and anion binding should weaken this repulsive interactions.[41,42] Still, the exact interaction mechanism between salts and the protein remains to be elucidated.[14] Furthermore, there is lack of clear thermodynamic understanding of the relative contributions from the salt-dependent protein–solvent interactions (μ_2^0) and protein–protein interactions (γ_2) on protein solubility for a net-charged protein.[43] Because of these limitations, it is challenging to pinpoint a complete thermodynamic picture of salt effects on the antibody solubility at pH 5.3.

Recently, Annunziata et al.[43] have shown for the positive-charged lysozyme at pH 4.5, its solubility drop is mainly dependent on the weakening of repulsive electrostatic protein–protein interactions by anion binding, even up to 0.25 M NaCl. Furthermore, it has been demonstrated that the decrease in lysozyme solubility in salt solutions at the pH conditions below its pI was closely correlated with salt's perturbations on protein–protein interactions as measured by LLPS.[44] Specifically, weakened repulsive electrostatic intermolecular interactions by salts lead to a decrease in protein solubility.[44,45] For this antibody, the anion-dependent solubility decrease is consistent with the observation that the intermolecular interactions measured by LLPS became less repulsive in the KCl and KSCN solutions up to approximately 100 mM at pH 5.3.[15] Therefore, the anion-modulated repulsive electrostatic protein–protein interactions may be a key thermodynamic factor at determining the antibody solubility at pH 5.3 as the salts are added initially.

Although the general trend of antibody solubility drop by salts may be explained by Debye screening[9] (salt ions screen the repulsive electrostatic interactions), it does not explain the observed anion specificity for reducing the antibody solubility at the ionic strength below approximately 50 mM. The reason is that Debye screening simply treats different monovalent ions equally as a single point charge.[9] Therefore, our observation suggests that the monovalent anions display specific interactions with the antibody. Namely, SCN^- has the strongest binding with the antibody and most effectively decreases the Q^*, weakens the repulsive electrostatic interactions, and reduces the antibody solubility, followed by Cl^- and F^-. This idea is consistent with our experimental results from the Q^* and zeta-potential measurements, and also supported by our previous finding from the LLPS study for this antibody.[15] The ranking for the binding strength between the anions and antibody is also in agreement with what has been observed for other positive-charged proteins including antibodies,[46] BSA,[37] and lysozyme[10,41] in monovalent salt solutions. In addition, our observed ranking for the binding strength conforms to the "law of matching water affinity," which predicts that chaotropic (weakly hydrated) SCN^- can interact more strongly to the net positive-charge antibody than the strongly hydrated (kosmotropic) anions, such as F^-, because the positive charges on the antibody are from the weakly hydrated side chains of lysine, arginine, and histidine. Another evidence to support the rankings of the anion binding strength is found in which the antibody solubility minimums in the three salt solutions are reached at different ionic strength. Specifically, the more strongly a monovalent anion binds to the antibody, the lower anion amount is required to saturate the binding sites.

Another important finding in our experiment is that the solubility minimum in the KSCN series at pH 5.3 is much lower than those in the KCl and KF series. The solubility behavior agrees with the trends of the antibody–antibody interactions as revealed by the LLPS data,[15] wherein the intermolecular interactions were more attractive in the KSCN series at pH 5.3 than in the KCl or KF series. Both observations indicate the presence of the additional attractive intermolecular interactions in the KSCN solution. Our explanations are supported by the finding of a Monte Carlo simulation, revealing that the addition of chaotropic (or polarizable) ions, such as SCN^-, introduces this additional interaction of dispersion force in nature between protein molecules.[47]

Anion-Specific Salting-In of the Antibody After Charge Neutralization

At pH 5.3 for all three anions studied, the solubility began to increase (salting-in) after a minimum was attained. This transition was seldom reported in protein solubility measurement in monovalent salt solutions. What appears to occur after charge neutralization is the salting-in event, most likely mimicking the antibody solubility behavior in the three salt solutions at pH 7.1, where the net charge is approximately zero. In another word, after the completion of charge neutralization by the anions, the antibody acts effectively as a pseudo charge-neutral species and the repulsive electrostatic intermolecular interactions are minimized. Therefore, the effectiveness of the three anions for increasing the solubility should follow the order of $SCN^- > Cl^- > F^-$, similar to that at pH 7.1. This is consistent with our results, as shown in

Figure 5a. This salting-in event agrees with the trend of protein–protein interactions becoming less attractive perturbed by the salts from our LLPS work.[15]

In Figure 5b at pH 6.1, the above transition from charge neutralization to the salting-in event was less dramatic than at pH 5.3 because the antibody had less positive charge. The presence of a solubility minimum at approximately 30 mM in the KSCN solubility–ionic strength relationship suggested that less SCN^- was needed to neutralize the smaller net charge at pH 6.1 than at pH 5.3. Despite the use of phosphate as the buffer salt at pH 6.1, the decrease in solubility by the addition of KSCN suggests that net charge was not completely neutralized by the phosphate used as a buffer reagent. The salting-in effect then begins to dominate after reaching the solubility minimum. We do not have enough solubility data to demonstrate the nonmonotonic transition in either KCl or KF solutions. However, the less dramatic initial rise of solubility below 100 mM KCl at pH 6.1 versus pH 7.1 in Figures VIa and VIb in Supporting Information suggests that the charge neutralization effect pre-empts the salting-in effect to a certain degree.

It is interesting to note that the salting-in trends of the antibody at pH 5.3 for the three monovalent anions were not observed for lysozyme at pH conditions below its p*I* using similar monovalent salts. For lysozyme, no solubility increase was observed for the KSCN tested up to 0.5 M at pH 4.5.[19] In another study of lysozyme solubility, a wider pH was covered at different ionic strength with NaCl, and no increase in protein solubility was observed up to 1.2 M NaCl for the pH conditions between 3.3 and 8.7.[39] The above results may be explained by charge neutralization, similar to what we mentioned previously in our LLPS study.[15] The surface charge density of lysozyme, that is, approximately 0.05 C/m^2 at pH 4.5, is high mainly because of its smaller size, in comparison with the value of 0.013 C/m^2 for this antibody at pH 5.3. The absence of a solubility minimum for lysozyme with 0.5 M KSCN at pH 4.5 suggests that KSCN had not completely neutralized the net charge of lysozyme before the salting-in event occurred. Other monovalent anions with weaker binding than SCN^- would have even less chance to neutralize the net charge of lysozyme. This perhaps explains why the onset of increased solubility did not occur for any of the other monovalent anions within the ionic strength conditions tested for lysozyme.

CONCLUSIONS

The solubility of a recombinant monoclonal antibody was examined to ascertain the influence of ionic strength and salt type at three different pH conditions using three monovalent salts—KF, KCl, and KSCN—at ionic strength conditions below 0.7 M. The solubility behavior was complex, exhibiting both monotonic and nonmonotonic trends, depending on salt concentration, salt type, and pH. We propose the following explanations for how monovalent anions affect the antibody solubility at pH conditions below the p*I* at ionic strength below 0.7 M. Initially, the antibody solubility behavior is influenced by the anion's weakening effect on repulsive electrostatic intermolecular interactions. The anions bind to the net positive-charge antibody surface, neutralize the net charge of the protein, resulting in weaker electrostatic repulsive interactions. As a result, the protein solubility decreases as the ionic strength increases, with the effectiveness of the anion following the order of $SCN^- > Cl^- > F^-$ for precipitating the antibody. After the anions complete the charge neutralization, salting-in of the antibody takes place, possibly still through the anion's binding to a pseudo charge-neutral antibody–anion complex. The effectiveness of the anion follows the order of $SCN^- > Cl^- > F^-$ for increasing the antibody solubility. Under a pH condition close to the p*I*, a similar salting-in behavior was observed.

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